Extraction of Antioxidant Compounds from Blueberry Fruit Waste and Evaluation of Their In Vitro Biological Activity in Human Keratinocytes (HaCaT)

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Abstract

The purpose of this study was to investigate the biological properties of an extract obtained from the waste of blueberry fruit. The study covered the optimization of extraction of antioxidants from blueberry pomace and the determination of antioxidant properties of the extract using HaCaT as the model organism. Research showed that the yield of antioxidants extraction from blueberry waste was dependent on the applied extraction conditions. Based on the mathematical models, the optimal conditions of extraction process in which the maximum quantity of antioxidant compounds is achieved from the waste mass unit, i.e., the relation of the waste mass to the volume of ethanol equal to 1:17.36, and process time equal to 1000 s. The obtained extract was characterized by high antioxidant activity, which was shaped by high content of polyphenols, mainly anthocyanins. Moreover, the extract showed a high ability to protect HaCaT cells from the occurrence of oxidative stress induced by H_2O_2. Cells treated with the extract and H_2O_2 generated a lower amount of ROS than cells treated with H_2O_2 only. The obtained results will be base of further studies on applying the extract in production of diet supplements and functional foods with increased antioxidant activity. Moreover, the main research material is blueberry pomace which is a troublesome waste material for juice producers. Consequently, according to a sustainable development idea, the study results will provide an opportunity to increase interest in the problem of rational use of the waste material to a certain extent.

Keywords Antioxidants · Bioactivity · Blueberry · Extraction · HaCaT · Pomace

Introduction

It is common knowledge that a well-designed diet that supplies adequate amounts of fruit and vegetables is an important way to prevent the development of cardiovascular conditions, neurodegenerative diseases, cancers, diabetes, and other lifestyle diseases. Scientific contributions indicate that the root cause of many pathological conditions is changes in human cells which are caused by reactive oxygen species. It is, therefore, important to look for new sources of antioxidants, which can effectively neutralize free radicals when these are produced in excess.

Berries, including blueberries, have a high antioxidant potential as these fruit contain plenty of polyphenol compounds, mainly anthocyanins (You et al. 2011). Polyphenols have the capacity to bind free radicals, thus minimizing the oxidant modifications to cellular components; such modifications impair the function of cells, causing them to mutate and die. In addition, polyphenol compounds chelate the ions of metals that initiate oxidation processes (Routray and Orsat 2011; Norberto et al. 2013). It has also been found that cyanidin isolates have a high capacity to inhibit the growth of cancer cells and to interfere with cellular metabolic pathways, resulting in the development of, for example, breast cancer, lung cancer, or promyelocytic leukemia (Olsson et al. 2004). Research also shows that the antiproliferative activity of anthocyanins is more than twice as high as that of other

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flavonoids and phenolic acids in relation to certain lines of cancer cells (Yi et al. 2005). Moreover, many scientific contributions indicate that anthocyanins from blueberry fruit have strong anti-inflammatory, antithrombotic, antimicrobial, anti-biofim, and antiadhesive properties, as well as a high capacity to reduce LDL cholesterol levels (Seeram et al. 2006; Routray and Orsat 2011; Khalifa et al. 2015; Silva et al. 2016).

Polyphenol compounds can be found virtually in all the anatomical parts of the blueberry. However, the highest concentration of these compounds is observed in the skin of the blueberry fruit (Matiacevich et al. 2013). Interestingly, the skin is often a waste product of intensive processing of the fruit, e.g., in juice making processes. It has been proved in many studies that the antioxidant potential of and fiber content in waste products from fruit processing are a few times higher compared to the juice made from the fruit or the whole fruit (Rodriguez-Mateos et al. 2014; Michalska and Łysiak 2015). Therefore, it seems necessary to find reasonable ways to utilize such waste. One way to make use of the waste is to make extracts that can be used in the manufacture of pharmaceuticals, functional foods, and cosmetics.

The purpose of this study was to examine the biological properties of an extract obtained from the waste of blueberry (*Vaccinum corymbosum* L.) fruit. The study covered the optimization of the extraction of bioactive compounds from the waste from blueberry fruit using RSM; to determine the profile of polyphenol compounds and the antioxidant activity of the extract; and to prove the antioxidant properties of the extract with the use of immortalized human keratinocytes (HaCaT cells) as the model organism.

Materials and Methods

Research Material

Fresh blueberry (*Vaccinum corymbosum* L.) fruit of the Blue-crop variety was harvested in July 2020, from a commercial plantation located in Strzyzow in south-eastern Poland. Blueberries were fully colored, ready for consumption, without mold infestation and mechanical damage. Blueberry waste was obtained by processing the juice from the fruit (1.5 kg) using a Philips slow juicer HR1945/80 (Amsterdam, Denmark). The juice was discarded, and the blueberry waste was lyophilized. Before the antioxidant extraction, blueberry waste was ground to an average particle size of 1 mm.

Optimization of Ultrasound-Assisted Extraction (UAE) Using Response Surface Methodology (RSM)

Optimization of extraction process was performed by means of a RSM, using two-factor, three-level compositional plan. An influence of the ratio of blueberry waste mass to ethanol volume (x_1) and time of process (x_2) on the total yield of extraction (y_1), the polyphenol recovery (y_2), and the antioxidant activity of dried extract (y_3) was examined in this research (Piechowiak et al. 2020a).

The antioxidant compounds were extracted from blueberry waste using a batch extractor equipped with SONOPULS sonicator HD 2070.2 and titanium probe MS 73 (BANDELIN electronic, Berlin, Germany) at an ultrasonic frequency of 20 kHz and a constant power of 70 W. In this method, 1 g of dry matter of blueberry waste was placed into an extraction vessel and sonicated with ethanol (96%, Chempur, Piekary Slaskie, Poland) according to the conditions presented in Table 1. The obtained crude extract was filtered in vacuum filtration apparatus (Merck Millipore, Burlington, MA, USA), evaporated, and dried in rotary evaporator at 45 °C and 300 mbar (Heidolph, Schwabach, Germany). Samples after extraction were subjected to analysis of total yield of extraction, total phenolic level (polyphenol recovery), and antiradical activity against DPPH (Piechowiak et al. 2020a).

Total Yield of Extraction

Total yield of extraction [g 100 g^{-1}] was determined using the following formula:

$$Y_E = \frac{w_e}{w_s} \times 100$$

where:
w_e: the weight of blueberry waste extract [g],
w_s: the weight of blueberry waste [g].

Chemical and Biochemical Analysis of the Extract

Antioxidant Activity with DPPH$^{\cdot}$

To 180 μl of 100 μM DPPH$^{\bullet}$ solution (Sigma-Aldrich, Steinheim, Germany), 20 μl of the extract was added. After 30 min of incubation in darkness, the absorbance was measured at 515 nm using EPOCH microplate reader (BIOTEK, Winooski, USA). Quercetin (Sigma-Aldrich, Steinheim, Germany) solutions (0–0.25 mg ml^{-1} in methanol) were used for calibration and the results were expressed as milligrams of quercetin equivalent (QE) per 1 g of the extract (Piechowiak et al. 2020a).

Antioxidant Activity with ABTS$^{\cdot+}$

To 180 μl of fresh prepared ABTS$^{\bullet+}$ solution (Sigma-Aldrich, Steinheim, Germany), 20 μl of the extract

Table 1 Central composite design of two variables with their observed responses ($\bar{x} \pm$ SE, n = 3)

Nr	Time [s]	Ratio of the blueberry waste mass to ethanol volume [g ml^{-1}]	Total yield of extraction [g 100 g^{-1}]	Polyphenols recovery [g GAE 100 g^{-1}]*	Antioxidant activity DPPH· [mg QE g^{-1}]**
	X_1	X_2	Y_1	Y_2	Y_3
1	1026	0.066	39.80 ± 1.41	4.18 ± 0.18	51.92 ± 2.63
2	1026	0.025	30.02 ± 0.71	2.73 ± 0.13	60.26 ± 1.86
3	605	0.046	25.89 ± 0.90	2.69 ± 0.24	62.95 ± 3.27
4	10	0.046	5.95 ± 0.57	0.53 ± 0.01	20.12 ± 2.39
5	184	0.025	12.41 ± 1.34	0.94 ± 0.01	36.93 ± 1.80
6	605	0.017	18.95 ± 0.89	1.51 ± 0.02	46.63 ± 2.06
7	605	0.046	25.27 ± 2.30	2.31 ± 0.03	59.77 ± 1.46
8	605	0.046	26.76 ± 2.81	2.38 ± 0.01	55.71 ± 3.21
9	1200	0.046	35.06 ± 0.64	3.15 ± 0.12	59.73 ± 2.44
10	184	0.066	12.45 ± 1.29	1.10 ± 0.05	53.04 ± 2.56
11	605	0.046	25.98 ± 1.93	2.45 ± 0.05	59.47 ± 0.72
12	605	0.046	26.18 ± 2.15	2.51 ± 0.06	60.01 ± 2.11
13	605	0.074	26.99 ± 1.37	2.62 ± 0.11	55.49 ± 3.30
Adjusted determination coefficient—*Adj*-R^2			0.98	0.93	0.88
Lack of fit test results, α = 0.05			p > 0.05	p > 0.05	p > 0.05

*The results expressed as gallic acid equivalent (GAE).

**The results expressed as quercetin equivalent (QE).

was added. After 30 min of incubation in darkness, the absorbance was measured at $\lambda = 400$ nm. Quercetin (0–0.25 mg ml^{-1}) was used for calibration and the results were expressed as milligrams of quercetin equivalent (QE) per 1 g of the extract (Piechowiak et al. 2020b).

Total Phenolic Content

To 20 µl of the extract, 80 µl of H$_2$O, 25 µl of Folin-Ciocalteu reagent (Sigma-Aldrich, Steinheim, Germany), and 50 µl of 20% Na$_2$CO$_3$ were added. After 30 min of incubation in darkness, the absorbance of the solution was measured at 700 nm. Gallic acid (Sigma-Aldrich, Steinheim, Germany) solutions (0–0.25 mg ml^{-1} in methanol) were used for calibration and the results were expressed as milligrams of gallic acid equivalent (GAE) per 1 g of the extract (Piechowiak et al. 2020a).

Sample Preparation for HPLC Analysis

The extract (100 mg) was dissolved in 10 ml of 50% methanol solution. Next, the mixture was vortexed and sonicated for 5 min. Before the chromatography analysis, the analytical sample was diluted with appropriate mobile phase (1:1) and centrifuged at 12,000 g for 5 min. The supernatant obtained was used for further analysis (Kołodziejczyk et al. 2013).

Qualitative Analysis of Anthocyanins Using LC–MS

The identification of anthocyanins was conducted using UHPLC system (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a DAD detector and mass spectrometer (MS, Q Exactive Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) (Sójka et al. 2016). The tested anthocyanins were separated on Gemini-NX C18 110 Å column (150 mm × 4.6 mm i.d., 3 mm) equipped with pre-column (4 mm × 3 mm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 1% (v/v) formic acid in water (mobile phase A), and 1% formic acid in methanol (mobile phase B). The gradient separation was performed as follows: 0–30 min, 20–65% (v/v) B; 30–31 min, 65–100% (v/v) B; 31–33 min, 100% (v/v) B; 33–34 min, 100–20% (v/v) B; 34–45 min, 20% (v/v) B at a flow rate of 0.5 ml min^{-1}. The column temperature was set 35 °C and injection volume was 10 µl. The mass detector parameters were as follows: vaporizer temperature of 400 °C, ion spray voltage of 3.8 kV, capillary temperature of 380 °C, and sheath gas and auxiliary gas flow rates of 60 and 20 units, respectively. The detector was operated in either the full MS or full MS/dd-MS2 scan modes. In the full MS mode, the scan range of m/z 250–1000 was used. To generate MS2 data, we set the NCE (normalized collision energy) parameter to 20 eV. Tuning and optimization were performed using the direct injection of cyanidin-3-glucoside

standard diluted in a 75:25 (v/v) mixture of mobile phases A and B at a flow of 0.25 ml min^{-1}. Identification of anthocyanins was performed based on the following standards: cyanidin-3-glucoside, delphinidin-3-glucoside (Extrasynthese, Genay, France), MS data, and available literature (Kucner et al. 2013; Chung et al. 2016; Li et al. 2016; Pertuzatti et al. 2016).

Quantitative Analysis of Anthocyanins and Other Polyphenols

The quantitative analysis of anthocyanins and sum of flavonols and chlorogenic acid in extract was performed according to the method presented by Sójka et al. (2016). The tested compounds were separated using the same chromatographic conditions. A Gemini 5u C18 110 Å column with dimensions of 150 mm×4.6 mm i.d was used for separation (Phenomenex, Torrance, CA, USA). The KNAUER Smartline (Berlin, Germany) chromatograph composed of a degasser (Manager 5000), two pumps (P1000), autosampler (3950), thermostat, and PDA detector (2800) was used. Chromatographic data was collected with ClarityChrom v. 3.0.5.505 (Knauer, Berlin, Germany). Standard curves using external standards of cyanidin-3-glucoside and quercetin-3-glucoside (both from Extrasynthese, Genay, France) and chlorogenic acid (Sigma-Aldrich, Steinheim, Germany) were used for quantification. Quantitative results of the determinations are given as cyanidin-3-glucoside equivalents for anthocyanins, and as quercetin-3-glucoside equivalents for flavonols.

Flavanol Analysis

The quantification of analysis of polymeric flavan-3-ols was carried out by acid-catalyzed degradation of polymeric proanthocyanidins in excess of phloroglucinol, as describe by Kennedy and Jones (2001) with minor modifications. A detailed description of the modifications was described in our previous publication (Sójka et al. 2013). For the phloroglucinolysis, 20 mg of freeze-dried extract was used. The same analysis conditions (column, gradient, phases, standards) and FD ($\lambda_{ex}=278$ nm, $\lambda_{em}=360$ nm) detector (Shimadzu RF-10Axl) were used to separate the products of these reactions but a different chromatography system (Shimadzu, Tokyo, Japan) consisting of LC-20AD pump, DGU-20ASR degasser, CTO-10AS thermostat, and SIL-20AC autosampler. Concentrations of phloroglucinol adducts, i.e., (-)-epicatechnin-phloroglucinol and (+)-catechin-phloroglucinol, were calculated using (-)-epicatechnin-phloroglucinol
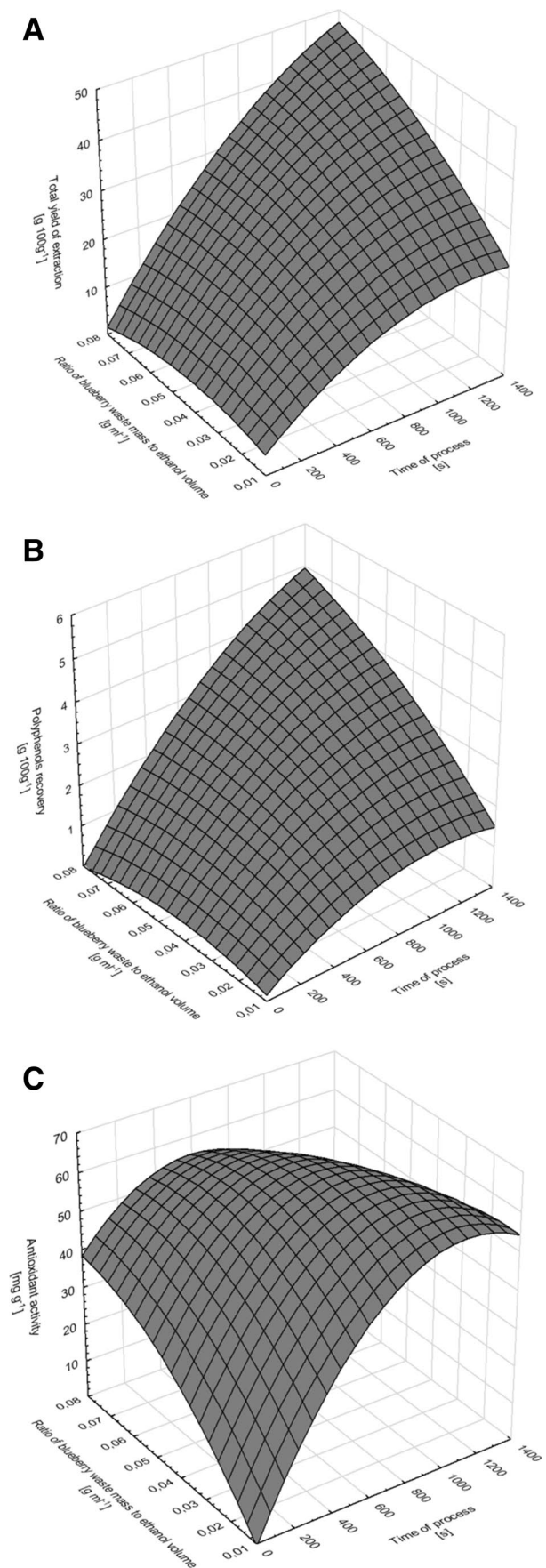
Table 2 Comparison of experimental data and results calculated based on regression models

Response Yi	Predicted value	Confidence interval		Experimental value $\overline{x} \pm SE$ (n = 3)
		95% min	95% max	
Y_1—total yield of extraction [g dried extract 100 g^{-1}]	36.67	35.00	38.64	36.72 ± 1.22
Y_2—polyphenols recovery [g GAE 100 g^{-1}]	3.32	3.30	3.93	3.45 ± 0.12
Y_3—antioxidant activity (DPPH·) [mg QE g^{-1}]	58.28	50.67	65.89	57.09 ± 2.52

standard curve. The concentration of terminal units, i.e., (-)-epicatechin and (+)-catechin, were calculated using (-)-epicatechin curve. In both cases used curves were prepared from procyanidin B2 standard (Extrasynthese, Genay, France) which was phloroglucinolysed according to the above procedure. The average degree of polymerization was measured by calculating the molar ratio of all the flavan-3-ol units (phloroglucinol adducts + terminal units) to (−)-epicatechin and (+)-catechin which correspond to terminal units. Free monomers of (−)-epicatechin and (+)-catechin were determined from extracts prepared in the "Sample preparation for HPLC analysis" section. The same HPLC conditions as for phologucinolysed samples were used, and the calculations were done from curves prepared from commercial standards of (-)-epicatechin and (+)-catechin (Sigma-Aldrich, Steinheim, Germany).

Determination of Antioxidant Activity of the Extract Using HaCaT Cell Line

Cell Culture

Human keratinocyte cell lines (HaCaT; ATCC CRL-2522) were obtained from the American Type Culture Collection (ATCC, distributor: LGS Standard, Łomianki, Poland). The cells were cultured in minimum essential medium (MEM), supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. Cells were cultured in humidified atmosphere with 5% CO_2 at 37 °C until it reached confluence. Further, cells were seeded in 96-well plates at a density of 5×10^3 cells per well in a 96-well plate (for H_2DCF-DA) or 9×10^5 cells per well in a 6-well plate (for catalase and superoxide dismutase activity and antiradical activity assays) and cultured for 24 h before experiment. Subsequently, the cells were treated with tested compounds, in contrary to vehicle-treated cells (control, vehicle = PBS) (Nizioł-Łukaszewska et al. 2020).

Table 3 Anthocyanin identification (LC–MS) in blueberry waste extract

Peak no	Retention time [min]	MS (m/z)	MS/MS (m/z)	Tentative identification
1	3.85	465.1	303.05	Delfinidin-3-galactoside
2	4.73	465.1	303.05	Delfinidin-3-glucoside
3	5.10	449.1	287.05	Cyanidin-3-galactoside
4	5.56	449.1	287.05	Cyanidin-3-glucoside
5	6.23	479.1	317.06	Petunidin-3-galactoside
6	7.03	479.1	317.06	Petunidin-3-glucoside
7	7.70	nd	nd	Unidentified anthocyanin
8	8.02	449.1	317.06	Petunidin-3-arabinoside
9	8.78	463.1	301.07	Peonidin-3-galactoside
10	9.22	493.1	331.08	Malvinidin-3-galactoside
11	10.22	493.1	331.08	Malvinidin-3-glucoside
12	11.38	463.1	331.08	Malvinidin-3-arabinoside
13	15.07	nd	nd	Unidentified anthocyanin
14	15.75	535.1	331.08	Malvinidin-3-acetyl-hexoside
15	16.39	521.1	317.06	Petunidin-acetyl-hexoside
16	19.17	535.1	331.08	Malvinidin-3-acetyl-hexoside

Influence of Blueberry Waste Extract on ROS Generation

The 2′,7′-dichlorodihydrofluorescein diacetate probe (H_2DCF-DA, Sigma-Aldrich, St. Louis, MO, USA) was used as an indicator of ROS production after exposure to 10 ng ml^{-1} and 5 µg ml^{-1} solutions of blueberry extract per se and together with 0.003% hydrogen peroxide solution (H_2O_2). The procedure was performed according to Szychowski et al. (2019). The 5-µM solution of H_2DCF-DA was used to determine the ROS production in HaCaT cell line. Briefly, cells were seeded on a 96-well plate at 5×10^3 cells per well 24 h before experiment. Next, the H_2DCF-DA solution in serum-free medium was applied for 30 min. After this time, the medium was removed, and the cells were washed once with PBS. Subsequently, the chosen concentrations of blueberry extract per se and together with 0.003% H_2O_2 were added to cells. The measurement of fluorescence was performed after 1, 24, and 48 h after studied compounds treatment at maximum excitation 485 nm and emission spectra 535 nm, using a microplate reader. The results were expressed as percentage of control (vehicle-treated cells). Cells treated with 0.003% H_2O_2 were used as a positive control.

Antioxidant Enzymes Activity

HaCaT cells were seeded on a 6-well plate, 24 h before the experiment. After this time, the medium was removed and replaced with working solutions of blueberry extract (10 ng ml^{-1} and 5 µg ml^{-1}) and 0.003% H_2O_2 for 24 and 48 h. Subsequently, medium was removed, cells were washed 3 times with PBS, scraped in the presence of Tris buffer (pH = 7.2) and immediately frozen in −80 °C. Shortly before the experiment, samples were thawed and the protein concentration was determined by Bradford method (Kruger 1994). Subsequently, SOD activity was determined using colorimetric method, according to Ukeda et al. (1997) protocol, which based on the measurement of the level of inhibition of nitro blue tetrazolium (NBT) reduction by SOD in xanthine/xanthine oxidase system. Briefly, 100 µl of 50 mM sodium phosphate buffer (pH = 7.4), 25 µl of 5 mM xhantine (Sigma-Aldrich, St. Louis, MO, USA), 50 µl of 5 mM NBT (Sigma-Aldrich, Steinheim, Germany), and 25 µl of cell lysate were added to a 96-well plate. The reaction was initiated by addition of 5 µl of diluted (10 times) xhantine oxidase from bovine milk (5 U mg^{-1} of protein, Sigma-Aldrich, St. Louis, MO, USA). The kinetics of the absorbance was measured for 20 min at 490 nm at 35 °C using a microplate reader. The catalase activity was assayed according to the method presented by Hadwan et al. (2018) with minor modification. Briefly, 10 µl of cell lysate and 50 µl of 50 mM sodium phosphate buffer (pH 7.4) were added into a

96-well plate. The reaction was initiated by adding 20 µl of 10 mM H_2O_2 per well and stopped after 2 min of incubation at room temperature by adding 50 µl of 50 mM ammonium metavanadate solution (Chempur, Piekary Śląskie, Poland). The absorbance was measured at 465 nm. The obtained results of catalase and superoxide dismutase activity were normalized to total protein content (Bradford methodology) and expressed as percentage of control (vehicle-treated cells) (Kruger 1994).

Table 4 Antioxidants composition of blueberry waste extract

Total antioxidant activity of blueberry waste extract	
ABTS$^{\bullet+}$ [mg QE g^{-1}]*	88.24 ± 1.98
DPPH$^{\bullet}$ [mg QE g^{-1}]*	60.85 ± 2.75
Total phenolic content [mg GAE g^{-1} d.m.]**	312.42 ± 6.03
Anthocyanins [mg g^{-1} extract]***	
Delfinidin-3-galactoside	6.32 ± 0.06
Delfinidin-3-glucoside	5.06 ± 0.05
Cyanidin-3-galactoside	6.63 ± 0.07
Cyanidin-3-glucoside	0.82 ± 0.01
Petunidin-3-galactoside	5.26 ± 0.05
Petunidin-3-glucoside	5.36 ± 0.07
Unidentified anthocyanin	0.44 ± 0.01
Petunidin-3-arabinoside	3.37 ± 0.04
Peonidin-3-galactoside	0.75 ± 0.01
Malvinidin-3-galactoside	11.76 ± 0.12
Malvinidin-3-glucoside	11.59 ± 0.10
Malvinidin-3-arabinoside	11.09 ± 0.12
Unidentified anthocyanin	0.27 ± 0.01
Malvinidin-3-acetyl-hexoside	0.67 ± 0.01
Petunidin-acetyl-hexoside	0.68 ± 0.01
Malvinidin-3-acetyl-hexoside	2.21 ± 0.03
Total anthocyanins	72.27 ± 0.75
Flavanols [mg g^{-1} extract]***	
Total flavanols	15.29 ± 0.51
(+)-Catechin	0.066 ± 0.00
(-)-Epicatechin	nd
Proanthocyanidins	15.23 ± 0.53
%extCat	0.50 ± 0.00
%extEpi	90.90 ± 0.06
%tCat	5.50 ± 0.00
%tEpi	3.10 ± 0.06
mDP	11.70 ± 0.06
Other polyphenols [mg g^{-1} extract]***	
Chlorogenic acid	1.84 ± 0.01
Total flavonols	14.10 ± 0.12

*The results expressed as quercetin equivalent (QE).

**The results expressed as gallic acid equivalent (GAE).

***Values are means ± standard errors (SE). n = 3. *%extCat*, percent of extender (+)-catechin; *%extEpi*, percent of extender (-)-epicatechin; *%tCat*, percent of terminal (+)-catechin; *%tEpi*, percent of terminal (-)-epicatechin; *mDP*, mean degree of polymerization.

Total Antiradical Activity of Cell Lysate Against ABTS[•+]

In order to determine the antiradical activity of cell lysate, the cell suspension (25 μl) was diluted with chilled PBS buffer (75 μl) contained 0.1% Triton X-100 and sonicated on ice for 30 s. The cell lysate was centrifuged at 10,000 g for 15 min at 4 °C and the supernatant was subjected to analysis. Next, to 10 μl of cell lysate, 90 μl of diluted ABTS[•+] solution ($A_{400} = 1.0$; prepared from 7 mM ABTS in 2.45 mM potassium persulfate) was added. After incubation in darkness at room temperature for 30 min, the absorbance was measured at 400 nm. The obtained results were normalized to 1 mg of protein and expressed as trolox [μg] equivalent activity per 1 ml of cell lysate (Piechowiak et al. 2020b).

Statistical Analysis

The data presented in this manuscript are mean values with standard error (SE). Each variant of extraction (based on central composite design, Table 1) were performed in triplicate ($n = 3$). This data were analyzed using multiple regression and ANOVA with Design Expert 13.0. software (Statease, Minneapolis, MN, USA). The antioxidant activity and phenolic compound level in the extract were analyzed three times ($n = 3$). The intracellular ROS level, antioxidant enzymes' activity (CAT and SOD), and antioxidant activity of cell lysates were determined six times for each tested substances ($n = 6$). This data were subsequently used in statistical analysis. The significance of differences was performed using one-way ANOVA and the Tukey test ($\alpha = 0.05$) and denoted as * < 0.05, ** < 0.001, and *** < 0.001. The statistical analysis was performed with STATISTICA 13.0 PL.

Results and Discussion

Optimization of Extraction Process of Antioxidants from Blueberry Waste

The efficiency of recovery of bioactive substances from plant raw materials is strictly dependent on the conditions of the extraction process, i.e., the extraction method, type and amount of solvent, process temperature, mixing intensity, and operations preceding the actual extraction, e.g., the degree of fragmentation or humidity (Jin et al. 2011). Numerous scientific studies also show that the inclusion of ultrasound in batch extraction increases the degree of damage to the cell walls, resulting in better penetration of the solvent into the matrix, and consequently increasing the efficiency of the process, as well as minimizing solvent consumption and shortening extraction (Chemat et al.

2017). Therefore, ultrasound-assisted batch extraction was used to recover bioactive substances from blueberry waste.

In the presented work, we used the response surface method to study the extraction process and determine the optimal process conditions. The RSM method is a set of mathematical and statistical tools that enable determination of the influence of many factors on the dependent variables, mutual interactions between factors and also of the optimal parameters of a given process based on regression analysis and analysis of variance (Piechowiak et al. 2020a).

Table 1 shows the RSM experimental plan and the results in the efficiency of extraction, recovery of polyphenols and antioxidant activity of dry extract. Based on the analysis of variance (ANOVA), we found that both the process time and the amount of solvent used had a significant impact on the values of all analyzed parameters. As the extraction time was extended, and the amount of ethanol was increased, we noted a gradual increase in the efficiency of extraction, recovery of polyphenols, and antioxidant activity. However, after exceeding a certain critical value (the maximum point on the graph), we noted a gradual decrease in the parameters tested (Fig. 1). The dependencies obtained are presented by means of suitable mathematical Eqs. (1–3). Regression analysis showed that the generated mathematical models were characterized by a good fit factor and no significance in the lack of fit test. This means that the above equations can be used to predict the efficiency of extraction:

$$Y_1 = 0.028x_1 + 183.56x_2 + 0.28x_1x_2 - 1.30 \bullet 10^{-5}x_1^2 - 2476.16x_2^2 \quad (1)$$

$$Y_2 = 10^{-2} \bullet 2.50 \bullet 10^{-3}x_1 + 24.73x_2 + 3.74310^{-2}x_1x_2 - 1.37 \bullet 10^{-6}x_1^2 - 307.72x_2^2 \quad (2)$$

$$Y_3 = x_1 + 1201.87x_2 - 0.71x_1x_2 - 4.80 \bullet 10^{-5}x_1^2 - 7136.15x_2^2 \quad (3)$$

Based on the results of the regression analysis and ANOVA, we have established the optimal conditions for the extraction process, in which the yield of bioactive compounds from blueberry waste and antioxidant activity reach maximum values (Table 2). The extraction process should be carried out for 1000 s, using 17.36 ml of ethanol per 1 g of pomace. In order to confirm the correctness of the adopted optimal conditions, we additionally conducted three experiments (under optimal conditions). We found that the obtained experimental data did not differ statistically significantly from the values calculated with the regression models. This confirms the correctness of the adopted regression models and the optimal conditions of ultrasound-assisted batch extraction.
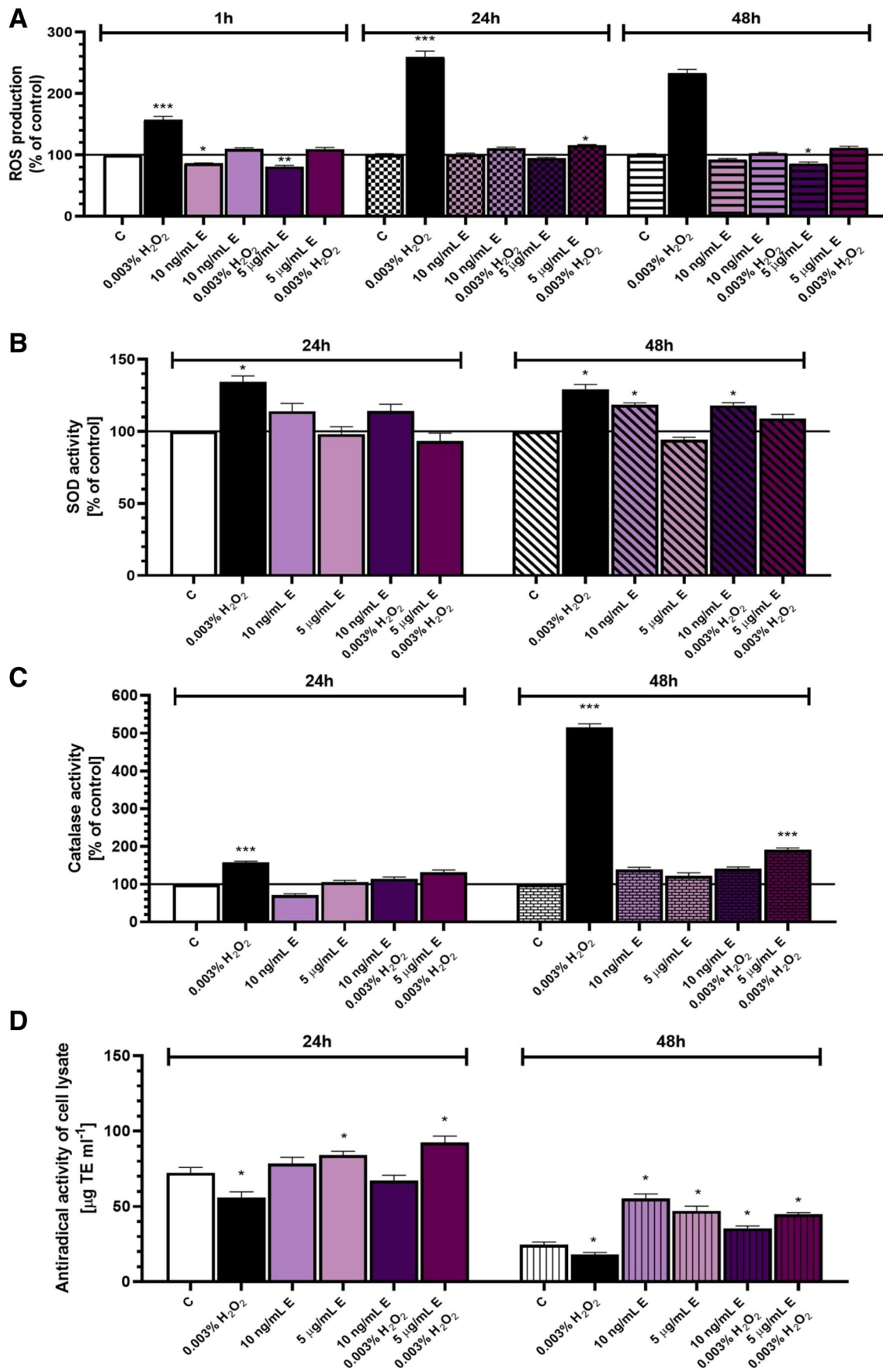
◄**Fig. 2** The effect of blueberry waste extract on the level of oxidative stress markers in HaCaT cell line. **A** The reactive oxygen species production. **B** The activity of superoxide dismutase. **C** The activity of catalase. **D** The antiradical activity of cell lysate. Mean values ($n=6$) with standard error (error bars) with *, **, and *** are statistically different from the respective control at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (one-way ANOVA, Tukey test). On the figure: E, blueberry waste extract; C, control sample

Antioxidants Composition of Blueberry Waste Extract

HPLC analysis revealed that the blueberry waste extract is a good source of polyphenols. In the course of our research, we identified four groups of polyphenol compounds belonging to flavonoids, namely anthocyanins, flavanols, proanthocyanidins, and flavonols as well as chlorogenic acid (Table 3, Table 4). We found that anthocyanins were dominant in the extract and its content was 72.27 mg g^{-1}. Moreover, the extract was characterized by high total antiradical activity against ABTS and DPPH and amounted to 88.24 and 60.85 mg of quercetin equivalent per 1 g of the extract, respectively.

In recent years, a number of research reports have been published on the impact of different extraction techniques on the recovery of bioactive compounds from berry waste as well as on the polyphenols profile and the antioxidant activity of obtained extract (Paes et al. 2014; Waterhouse et al. 2017; Lončarić et al. 2020). However, the extraction yields determined by other authors were explicitly lower than those obtained by us. It was probably due to both the variable content of bioactive substances in the plant (depending on the growing conditions of the plant) as well as the method of obtaining the pomace and the extraction conditions adopted (Laroze et al. 2010; Paes et al. 2014). For example, in the work of Lončarić et al. (2020), the profile of polyphenol compounds and the antioxidant activity of blueberry pomace extracts obtained by high-voltage electrical discharges, pulsed electric fields, and ultrasound-assisted extraction (in an ultrasonic bath) were determined. Regardless of the extraction method, the authors identified 18 polyphenols in the extracts, the dominant group of which were anthocyanins. However, the content of the individual substances was closely associated with the technique and the process conditions used. The authors also found that among the different extraction techniques they tested, pulsed electric field-assisted extraction (electric field strength: 20 kV, number of pulses: 100) allowed them to recover the largest amount of anthocyanins per unit of blueberry waste weight (1624.54 μg g^{-1} of blueberry pomace). In the case of ultrasonic extraction (at 35 kHz), the highest content of polyphenols was recorded in the extract obtained after 15 min of the process, at 80 °C with the use of 50% acidified ethanol (924.28 μg g^{-1} of blueberry pomace). Paes et al.

(2014) acquired bioactive compounds from bilberry (*Vaccinum myrtillus* L.) pomace using supercritical carbon dioxide as well as ethanol, water, and acidified water as factors enhancing the solubility of polar components. They found that the highest recovery of polyphenols (134 mg g^{-1} of the extract) can be achieved by using an extractant containing 90% CO_2, 5% H_2O, and 5% ethanol and carrying out the process at a pressure of 20 MPa. Waterhouse et al. (2017) in turn conducted the extraction of polyphenols from blueberry pomace, using enzyme preparations with pectinase and cellulase activity in an acidic environment at a temperature of 20 and 50 °C. The authors found that the application of enzyme preparations increased the efficiency of polyphenol extraction carried out at 20 °C from 3655.42 mg 100 g^{-1} to 4131.82 mg 100 g^{-1}, and at 50 °C from 3896.12 mg 100 g^{-1} to 4368.92 mg 100 g^{-1} of the extract.

The Effect of Blueberry Waste Extracts on the Antioxidant System in HaCaT Cells

The next part of our study was to determine the antioxidant properties of blueberry waste extract with the use of immortalized human keratinocytes (HaCaT cells) as the model organism. For this purpose, HaCaT cells were treated with a 0.003% concentration of hydrogen peroxide, and extract from blueberry waste at various concentrations (10 ng ml^{-1} and 5 μg ml^{-1}). The well-described indicators of oxidative stress in the human cells such as intracellular ROS production, antioxidant enzymes' activity (SOD, CAT), and antiradical activity of cell lysate were measured (Matés et al. 1999).

The results of our study show clearly that the extracts reduce the level of reactive oxygen species in cells treated with hydrogen peroxide throughout the cell culture period (Fig. 2A). The best protective effect occurred after 24 h of incubation. The level of ROS in the cells stressed with hydrogen peroxide increased by ~159% as compared to the reference sample, while the level of ROS in the cells treated also with the waste product extract was similar to that for the reference sample, regardless of the concentration. Moreover, the cells treated with the extract and not exposed to the activity of hydrogen peroxide produced less ROS than the control sample.

Antioxidant enzymes are the first line of defense against the toxicity of reactive oxygen species in eukaryotic cells. The superoxide dismutase enzyme catalyzes the dismutation of the superoxide radical into hydrogen peroxide, and this catalysis accelerates the decomposition of hydrogen peroxide into water (Matés et al. 1999). In vitro studies involving cell lines and yeast or in vivo studies involving animals or humans show a close correlation between the level of ROS and the activity of these enzymes. An increased level of ROS in a cell increases the activity of

appropriate enzymes involved in the neutralization of ROS (Matés et al. 1999; Piechowiak and Balawejder 2019; Yan et al. 2020). A similar correlation was found in the study under discussion. Superoxide dismutase (SOD) and catalase (CAT) activities in keratinocyte cells exposed to H_2O_2 increased after 24 h to ~ 34% and ~ 57%, respectively, and after 48 h to ~ 29% and ~ 415%, respectively, as compared to the reference sample (Fig. 2B, C). The application of the waste product extract eliminated the toxicity of hydrogen peroxide. After 24 h of incubation, SOD and CAT activities in the cells treated with the extract and exposed to H_2O_2 were comparable to those in the reference sample. However, after 48 h of incubation, CAT activity in these samples increased slightly, but it was still significantly lower than the value estimated for the cells treated with H_2O_2 only. The finding is that the antioxidants (polyphenols) contained in the extract directly reacted with an excess of reactive oxygen species induced by H_2O_2 and "spared" the natural antioxidative mechanisms, thus preventing the occurrence of oxidative stress. Our study of the antiradical properties of cell lysates in relation to synthetic radicals (ABTS) has confirmed that the treatment of a culture with the extract increases the capacity of the culture to reduce the level of ROS in cells (Fig. 2D). The antiradical activity of HaCaT increased slightly after the treatment of the culture with the extract from the blueberry waste. This may indicate that the polyphenols contained in the extract penetrate the cell membrane of the cell into the cytoplasm and that the capacity of the cell to neutralize the ROS induced by hydrogen peroxide increases (Yagi et al. 2013).

Conclusion

Research showed that the yield of antioxidants extraction from the blueberry waste was dependent on the applied extraction conditions. On the basis of the mathematic models, the optimal conditions of the process in which the maximum quantity of antioxidant compounds is achieved from the waste mass unit, i.e., the relation of the waste mass to the volume of ethanol equal to 1:17.36, and process time equal to 1000 s. The obtained extract was characterized by high antioxidant activity which was shaped by high content of phenolic compounds, mainly anthocyanins. Moreover, the blueberry waste extract showed a high ability to protect HaCaT cells from the occurrence of oxidative stress exposed to hydrogen peroxide. Cells treated with extracts and H_2O_2 generated a lower amount of ROS than cells treated with H_2O_2 only. It should be noted that the factors that limit the possibility of a positive effect of polyphenols on the human body are the transformation of polyphenols in the human digestive system and their absorption

rate. Therefore, in order to fully determine the biological properties of blueberry pomace extract, it is necessary to perform additional in vivo tests using appropriate model organisms.

Declarations

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